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# OXYGEN POISONING IN THE ANNELID *TUBIFEX TUBIFEX*. II. OSMOTIC PROTECTION[1]

JOANNE G. WALKER[2]

*Department of Physiology and Biophysics, University of Illinois, Urbana, Illinois 61801*

Walker (1970) studied the effects of hyperbaric oxygen on *Tubifex tubifex*, an Annelid normally living in environments with extremely low oxygen tensions. These animals were killed by exposure to four atmospheres absolute oxygen pressure for 15 hours but recovered from exposures of up to eight hours or when longer exposures were interrupted by a sufficient interval at atmospheric conditions (Walker, 1970). In the present study the ability of various agents to modify the toxic effects of oxygen on *T. tubifex* has been investigated.

## Materials and Methods

*T. tubifex* was exposed to four atmospheres absolute oxygen pressure by the procedures described previously (Walker, 1970). The water used in this study both for worm exposures and for preparation of reagents was tap water which had passed through activated charcoal filters and to which 0.05 g disodium dihydrogen ethylenediamine tetraacetic acid (versene, Hach Chemical Co.) per liter was then added.

The effects of various agents were determined by adding 0.5 ml of the test solution to each depression of the paraffin block which already contained 4.5 ml water and one worm. Addition of agents was made immediately before oxygen exposure except where otherwise noted. In some experiments the effect of complete removal of the environmental water after oxygen exposure and replacement with another medium was tested. When heat stress experiments were run worms were exposed to increased temperatures in a water bath but were returned to room temperature to score survival.

Worm responses were judged by the following criteria: the presence or absence of movement and the presence of damage visible without a microscope. Worms having progressive damage and showing no movement for several observation periods were scored as dead. Such worms either became a grey, opaque, motionless mass or disintegrated completely. An asterisk following data for per cent survival in the tables indicates that the value does not correspond to an actual observation time. Such data were obtained from a graph of the per cent survival versus time after oxygen exposure for the experiment in question.

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[2] Present address: Department of Pharmacology, College of Medicine, Ohio State University, 370 W. 9th Ave., Columbus, Ohio 43210.

## Results

### 1. *Protection by solutes*

The possibility that glycolysis might be inhibited by hyperbaric oxygen in *T. tubifex* was first considered. Worms in either one per cent glucose or in water were exposed to oxygen in two doses interrupted by various intervals. These preliminary glucose experiments were a portion of the dosage fractionation experiments reported previously (Walker, 1970). In one experiment glucose was added during the interval following the first oxygen exposure. In another experiment worms were exposed to oxygen in water, in one per cent glucose, in 2,4-dinitrophenol at a final concentration of $1 \times 10^{-4}$ *M* and in one per cent glucose plus $1 \times 10^{-4}$ *M* 2,4-dinitrophenol. The results of these experiments are presented in Table I.

Table 1

*Survival of* T. tubifex *as affected by addition of glucose and of dinitrophenol during fractionated oxygen exposures (16 worms per group)*

| Worm group | Oxygen treatment | | | Post-exposure survival (per cent) | |
|---|---|---|---|---|---|
| | First dose hours | Interruption hours | Second dose hours | 48 hours | 153 ± 5 hours |
| Water | 8 | 2 | 8 | 24.1* | 0.0 |
| 1% Glucose (added at start of first exposure) | | | | 100.0 | 100.0 |
| 1% Glucose (added at end of first exposure) | | | | 51.7* | 12.5 |
| Water | 10 | 4 | 8 | 12.5 | 9.0* |
| 1% Glucose (added at start of first exposure) | | | | 91.1* | 87.5 |
| Water | 8 | 6 | 8 | 75.0 | 68.8 |
| 1% Glucose (added at start of first exposure) | | | | 100.0 | 93.8 |
| DNP $1 \times 10^{-4}$ *M* (added at start of first exposure) | | | | 97.7* | 93.8 |
| 1% Glucose + DNP $1 \times 10^{-4}$ *M* (added at start of first exposure) | | | | 93.8 | 93.8 |

* By interpolation.

One per cent glucose had a striking protective effect on oxygen-treated *T. tubifex*. Glucose had some protective effect even when it was added after the first eight-hour oxygen exposure. Control experiments showed that 2,4-dinitrophenol at $1 \times 10^{-4}$ *M* was not lethal for unoxygenated *T. tubifex;* this concentration, reported to uncouple oxidative phosphorylation (Loomis and Lipmann, 1948), not only did not reverse the protective effect of glucose but appeared to have some protective effect of its own.

*T. tubifex* were also exposed to oxygen in one per cent Proteose-Peptone (Difco) during the dosage fractionation experiments. This medium is very favorable for the growth of microörganisms. Worm depressions containing Proteose-Peptone rapidly became contaminated and the worms had to be discarded. Proteose-Peptone nevertheless provided a protection comparable to that of glucose during the period before fouling.

The protective effect of both glucose and Proteose-Peptone was immediately apparent from the behavior of oxygen-exposed worms when they were removed from the oxygen chamber. Worms exposed to oxygen in glucose or Proteose-Peptone were moving normally and were not contracted, while worms exposed in water were highly coiled and almost motionless.

Since such different substances as glucose, Proteose-Peptone and 2,4-dinitrophenol all protected *T. tubifex* against the toxic effects of oxygen and particularly since a moderate NaCl concentration was produced in dissolving and adjusting the pH of the 2,4-dinitrophenol solution, the possible protective effect of NaCl was suggested. Three groups of worms were therefore exposed to oxygen for 16 hours

TABLE II

*Survival of* T. tubifex *following 18 hours of oxygen exposure in various concentrations of glucose or sodium chloride (16 worms per group)*

| Worm group | Post-exposure survival (per cent) | | | |
|---|---|---|---|---|
| | 25 ± 4 hours | 53 ± 1 hours | 94 ± 5 hours | 144 ± 3 hours |
| Water | 62.5 | 62.5 | 43.8 | 37.5 |
| Glucose 0.1 *M* | 87.5 | 87.5 | 87.5 | 75.0 |
| Glucose 0.01 *M* | 87.5 | 87.5 | 87.5 | 81.3 |
| Glucose 0.001 *M* | 62.5 | 50.0 | 43.8 | 31.3 |
| Glucose 0.0001 *M* | 56.3 | 56.3 | 37.5 | 18.8 |
| Water | 8.3 | 8.3 | 8.3 | 8.3 |
| NaCl 0.1 *M* | 100.0 | 31.3 | 31.3 | 31.3 |
| NaCl 0.01 *M* | 100.0 | 87.5 | 87.5 | 81.3 |
| NaCl 0.001 *M* | 18.8 | 18.8 | 18.8 | 18.8 |
| NaCl 0.0001 *M* | 46.6 | 46.6 | 46.6 | 46.6 |
| Water | 25.0 | 18.8 | 18.8 | 18.8 |
| NaCl 0.05 *M* | 100.0 | 100.0 | 100.0 | 100.0 |
| NaCl 0.02 *M* | 100.0 | 100.0 | 100.0 | 100.0 |
| NaCl 0.005 *M* | 68.8 | 55.5* | 43.8 | 43.8 |

* By interpolation.

in water, in one per cent glucose and in NaCl at a concentration osmotically equivalent to the one per cent glucose and their post-exposure survival scored. Survival of *T. tubifex* at 219 hours after oxygen treatment was 25 per cent for worms exposed to oxygen in water, 100 per cent for worms exposed to oxygen in one per cent glucose and 87.5 per cent for worms exposed to oxygen in NaCl osmotically equivalent to the glucose. Again the protective effect was clear from the worms' behavior on removal from the oxygen chamber. Worms exposed to oxygen in either glucose or NaCl were more active and appeared more like normal worms than did the worms exposed to oxygen in water.

To determine the concentration providing maximum protection, worms were exposed to an 18-hour oxygen dose in solutions of glucose or NaCl ranging in concentration from 0.1 to 0.0001 *M*. Results of these experiments are presented in Table II.

It is clear that maximum protection resulted from treatment with glucose solutions between 0.1 *M* and 0.01 *M*. The NaCl solution giving the greatest protection had a concentration of 0.01 *M*. Sodium chloride at 0.1 *M* offered equivalent protection during the first day after exposure, but its beneficial effect decreased markedly during the following day. It is of interest that this relatively high concentration of NaCl was beneficial even though one half of the control worms not exposed to hyperbaric oxygen died in this concentration of NaCl within two days. When the NaCl optimum was more sharply defined, optimum and equivalent protection occurred in 0.05 *M* and 0.02 *M* solutions. These results are presented graphically in Figure 1. It is interesting to note that one per cent glucose used in all other glucose experiments has a molarity of 0.056; NaCl which is osmotically equivalent to one per cent glucose has a molarity of 0.028.

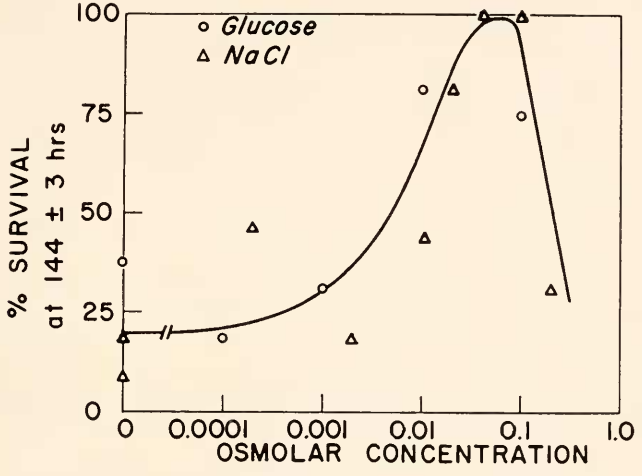


FIGURE 1. Survival of *T. tubifex* following 18 hours of oxygen exposure in various osmolar concentrations of glucose or sodium chloride.

Because of the marked protective effect of NaCl addition, it seemed essential to determine whether salts other than NaCl provided similar protection. Sodium nitrate and the chlorides of various monovalent and divalent cations were tested. All solutions were used at concentrations both above and below the osmotic equivalent of one per cent glucose. Complete dissociation of the salts at these concentrations was assumed. Results of these experiments are given in Table III.

These experiments indicate that protection against oxygen damage may be nonspecific in that salts with qualitatively different ionic composition had similar protective capacities. Calcium chloride at both concentrations and $MnCl_2$ at the lower concentration tested provided protection against hyperbaric oxygen equivalent to that produced by NaCl. Both concentrations of KCl and the higher concentrations of $CoCl_2$ and $MnCl_2$ were extremely toxic to the unoxygenated control worms. Most of the unoxygenated worms in the higher concentrations of KCl and $MnCl_2$ survived for two days but all were dead in five days. In 0.02 *M* KCl all the unoxygenated worms survived for six days but all were dead

TABLE III

*Survival of* T. tubifex *following 18 hours of oxygen exposure in various salt solutions (16 worms per group)*

| Worm group | Post-exposure survival (per cent) | | | | | |
|---|---|---|---|---|---|---|
| | 5 ± 1 hours | 18 ± 1 hours | 27 ± 3 hours | 42 ± 2 hours | 137 hours | 185 ± 1 hours |
| Water | 81.3 | 62.5 | 62.5 | 62.5 | 62.5 | 62.5 |
| NaCl 0.05 *M* | 100.0 | 100.0 | 100.0 | 100.0 | 93.4 | 93.4 |
| NaCl 0.02 *M* | 100.0 | 100.0 | 100.0 | 100.0 | 87.5 | 87.5 |
| KCl 0.05 *M* | 93.8 | 87.5 | 87.5 | 43.8 | 0.0 | 0.0 |
| KCl 0.02 *M* | 100.0 | 100.0 | 93.8 | 93.8 | 25.0 | 0.0 |
| $NaNO_3$ 0.05 *M* | 100.0 | 100.0 | 87.5 | 81.3 | 68.8 | 56.3 |
| $NaNO_3$ 0.02 *M* | 93.8 | 93.8 | 93.8 | 87.5 | 68.8 | 68.8 |
| $CaCl_2$ 0.02 *M* | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| $CaCl_2$ 0.002 *M* | 100.0 | 100.0 | 100.0 | 100.0 | 93.8 | 93.8 |
| Water | 100.0 | 25.0 | 25.0 | 18.8 | 18.8 | 18.8 |
| $CoCl_2$ 0.02 *M* | 100.0 | 62.5 | 0.0 | 0.0 | 0.0 | 0.0 |
| $CoCl_2$ 0.002 *M* | 100.0 | 43.8 | 6.3 | 6.3 | 6.3 | 6.3 |
| $MnCl_2$ 0.02 *M* | 100.0 | 75.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| $MnCl_2$ 0.002 M | 100.0 | 87.5 | 81.3 | 81.3 | 81.3 | 81.3 |

at nine days; in 0.02 *M* $CoCl_2$ half the unoxygenated worms were dead in six days. All of the unoxygenated control worms survived in all other salt solutions. It is all the more impressive, therefore, that even those salts which were eventually toxic to unoxygenated control worms delayed the death of oxygen-exposed worms during the first day following oxygen exposure. Sodium nitrate solutions produced a somewhat different effect. Protection by this salt was at first equivalent

TABLE IV

*Survival of* T. tubifex *as affected by replacement of the medium following 18 hours of oxygen exposure (16 worms per group)*

| Worm group | Post-exposure survival (per cent) | | |
|---|---|---|---|
| | 16 hours | 48 hours | 250 ± 12 hours |
| Water not replaced | 0.0 | 0.0 | 0.0 |
| Water replaced with water | 25.0 | 25.0 | 25.0 |
| Water replaced with 0.028 *M* NaCl | 81.3 | 86.8* | 62.5 |
| 0.028 *M* NaCl not replaced | 100.0 | 100.0 | 100.0 |
| 0.028 *M* NaCl replaced with 0.028 *M* NaCl | 100.0 | 100.0 | 100.0 |
| 0.028 *M* NaCl replaced with water | 93.8 | 93.5* | 81.3 |
| Water not replaced | 12.5 | 12.5 | 0.0 |
| Water replaced with water | 12.5 | 8.5* | 6.3 |
| Water replaced with 1% Glucose | 37.5 | 33.6* | 18.8 |
| 1% Glucose not replaced | 100.0 | 96.0* | 87.5 |
| 1% Glucose replaced with 1% Glucose | 100.0 | 100.0 | 81.3 |
| 1% Glucose replaced with water | 100.0 | 100.0 | 43.8 |

* By interpolation.

to the protection given by NaCl. This protective effect gradually decreased until at the termination of the experiment the $NaNO_3$-treated worms had a mortality equivalent to that of the controls.

Results in Tables II, III and V indicate that some worms in water survived after uninterrupted oxygen exposures of 16 to 18 hours. A variation in sensitivity of *T. tubifex* to oxygen was observed throughout these experiments which could not be strictly correlated with increasing storage time in the laboratory but may be related to the adaptation previously reported (Walker, 1970).

### 2. *Time factor in protection*

Exchange experiments were set up in an attempt to determine when osmotic protection from NaCl or glucose addition was most effective. Worms were given an 18-hour oxygen exposure in water which was withdrawn in a syringe immediately after oxygen exposure and replaced with 0.028 *M* NaCl. Other worms

TABLE V

*Survival of* T. tubifex *following addition of glucose or of sodium chloride at various times after 18 hours of oxygen exposure (16 worms per group)*

| Worm group | Post-exposure survival (per cent) | | |
|---|---|---|---|
| | 12 hours | 77 hours | 205 hours |
| Water addition 0 time | 60.0 | 53.4 | 33.7 |
| 1% Glucose addition 0 time | 87.5 | 62.5 | 50.0 |
| 0.028 *M* NaCl addition 0 time | 73.4 | 73.4 | 66.7 |
| 0.028 *M* NaCl addition ½ hour | 100.0 | 86.7 | 86.7 |
| 0.028 *M* NaCl addition 1 hour | 100.0 | 86.7 | 60.0 |
| 0.028 *M* NaCl addition 2 hours | 80.0 | 73.4 | 66.7 |
| 0.028 *M* NaCl addition 4 hours | 87.5 | 75.0 | 68.8 |
| 0.028 *M* NaCl addition 8 hours | 68.8 | 62.5 | 62.5 |
| 0.028 *M* NaCl addition 12 hours | 68.8 | 68.8 | 68.8 |

were exposed to oxygen in 0.028 *M* NaCl which was replaced with water after oxygen exposure. A similar experiment was run using one per cent glucose. Results of these experiments are shown in Table IV.

The presence of either saline or glucose during oxygen exposure resulted in a markedly increased worm survival. The continued presence of saline after removal from oxygen provided complete protection whether or not the saline was replaced by fresh saline solution. Even the replacement of saline with water after oxygen exposure did not greatly decrease worm survival. The presence of glucose after oxygen treatment greatly increased worm survival. Post-exposure replacement of glucose with water resulted in survival intermediate between the survival of worms treated continuously with water and worms in the continued presence of glucose. Addition of glucose after oxygen treatment may have a slight beneficial effect whereas addition of saline after oxygen exposure leads to markedly increased worm survival. Some worms which appeared to be moribund after exposure to oxygen in water resumed activity several hours after saline addition. Oxygen damage, however, was too great to be reversed completely and they eventually died.

The protective effect of post-exposure glucose and saline addition was substantiated by additional experiments; the data are presented in Table V.

Since addition of saline immediately following oxygen exposure resulted in a marked increase in worm survival, the next experiment was designed to test whether saline addition later in the recovery period would provide any protection and to determine at what time during the recovery period the saline addition would become ineffective. One trial was also run to determine whether treatment of the worms with saline (0.05 *M*) during the two hours previous to oxygen exposure in water had any beneficial effect.

Worms in 4.5 ml water were exposed to oxygen for 18 hours. A 0.5 ml volume of water or saline or glucose was added to each worm depression at the time of removal from oxygen or, in the case of saline, at various time intervals up to 12 hours after oxygen exposure. The final glucose concentration was one per cent; the final saline concentration was osmotically equivalent to that of the glucose.

The results, shown in Table V, indicate that saline addition as late as 12 hours after oxygen exposure enhanced survival. Glucose addition immediately after oxygen exposure resulted in survival intermediate between that of the controls exposed in water and that of worms treated with saline.

In another experiment a two-hour saline pretreatment had no protective effect; survival was indistinguishable from that of worms receiving no saline pretreatment.

### *3. Protection against other types of stress*

The foregoing experiments showed that there is little specificity regarding the ions involved in protection against oxygen poisoning. It appeared equally important to determine whether protection is specific for oxygen poisoning or whether increasing the osmotic concentration of the environment would reduce mortality following other types of stress as well. To test this possibility, heat and hydrogen peroxide were used as stressing agents.

(a.) *Heat stress.* In preliminary tests worms heated for five minutes in a water bath at 45° C exhibited, within a few minutes, a typical pattern of response: marked hyperactivity followed by segmental constriction and segmental rupture which soon ended in worm disintegration. A five minute exposure at 38° C had no detectable effect other than to stimulate worm activity. Worms heated at 37° C for 60 to 75 minutes exhibited the constriction-rupture-disintegration response sequence spread over several days. Thus, prolonged exposure to 37° C offered more accurate visualization of the stress effects and appraisal of possible protective capacities of saline addition. In subsequent experiments, each worm was placed in either 5 ml water or 5 ml 0.05 *M* NaCl in a test tube. The tubes were heated in a water bath at 37° C for 75 minutes and subsequent responses of the worms were observed.

During the first day following the heat stress, worms in water without added salt exhibited a high mortality. Only 14.4 per cent of the heat-treated worms survived after 21 hours; none were alive at 103 hours. This response was not unlike the typical response following a fairly high dose of oxygen. Saline treatment resulted in a marked initial protection; 92.9 per cent of the worms exposed to heat stress in a saline environment were alive after 21 hours. Most of the

saline-protected worms eventually died, however, so it appears that heat stress is not strictly comparable to oxygen damage at these dosages.

(b). *Hydrogen peroxide stress.* Hydrogen peroxide exposure was used as another type of stress. Preliminary experiments indicated that 20 $\mu$g per ml was a suitable hydrogen peroxide dose. Twenty four worms were therefore treated with 20 $\mu$g per ml hydrogen peroxide in combination with 0.05 *M* NaCl and their responses were compared to those of worms in hydrogen peroxide alone. After 640 hours 29 per cent of the worms in $H_2O_2$ and 78.3 per cent of the worms in $H_2O_2$ in combination with NaCl were alive. These data suggest that saline addition in combination with hydrogen peroxide has a beneficial effect on worm survival.

## Discussion

It is difficult to account for the protective capacities of such a widely diversified collection of compounds as glucose, Proteose-Peptone, 2,4-dinitrophenol, NaCl, $CaCl_2$, KCl, $MnCl_2$, $CoCl_2$ and $NaNO_3$. Various ions, particularly divalent cations, have previously been reported to have a protective effect in oxygen poisoning. Magnesium *in vitro* (Dickens, 1946a) and *in vivo* (Wittner, 1957), manganese *in vitro* (Dickens, 1946a; 1946b) and *in vivo* (Wittner, 1957; Gerschman, Gilbert and Frost, 1958b) and cobalt *in vitro* (Dickens, 1946a; Horn, Williams, Haugaard and Haugaard, 1967) and *in vivo* (Wittner, 1957; Gerschman, Gilbert and Caccamise, 1958a; Gerschman *et al.*, 1958b) all were found to decrease the toxic effects of oxygen.

Several reports of protection by salts against other agents similar to the protection found in the present study have appeared in the literature. Freese, Bautz-Freese and Bautz (1961) found that when increasing concentrations of NaCl were present inactivation of phage $T_4$ by the mutagenic agent hydroxylamine was inhibited. Saier and Giese (1967) reported that the changes occurring in *Paramecium multimicro-nucleatum* caused by exposure to ultraviolet irradiation were inhibited or delayed as the salt content of the medium was increased. Some protection was obtained when glucose solutions osmotically equivalent to the salt solutions were tested. The medium providing the best protection was hypotonic to the organism but high in calcium and magnesium.

*T. tubifex* is a freshwater dweller; the tap water used in the present study containing the disodium salt of versene at an effective osmotic concentration of $4.5 \times 10^{-4}$ *M* without other added solutes therefore corresponds closely to the worms' natural environment. Palmer (1968) reported that 10 per cent sea water (equivalent to 0.058 *M* NaCl; Prosser, 1961) was the highest salinity tolerated by *T. tubifex* without gradual acclimatization. Above this concentration worm deaths occurred in 24 hours. In the present study, NaCl at 0.1 *M*, the only concentration above 0.05 *M* tested, caused deaths among unoxygenated control worms also. Thus the highest salinity tolerated by *T. tubifex* corresponds closely to the saline concentration giving maximum protection against hyperbaric oxygen and the other stressing agents employed in the present study.

Palmer (1968) suggested that in higher salinities *T. tubifex* might be expected to require less energy expenditure to maintain itself hypertonic than in dilute environments causing continual tissue dilution. Such an argument could also be

applied to explain the present findings of solute protection during oxygen poisoning. Palmer's experiments (1968) however did not support her hypothesis; she found no change in the oxygen consumption of *T. tubifex* in environments from zero to 20 per cent sea water.

The rather narrow concentration range for maximum protection indicates that the osmotic concentration of the environmental medium is an important factor in the response of *T. tubifex* to oxygen. The protective effect of added ions appears to have little specificity and to be independent of the ionic composition of the salt. The enhanced survival observed in these experiments following oxygen exposure of *T. tubifex* in the presence of ions or glucose indicates that almost complete protection can be obtained against these doses of oxygen in this organism. The protection provided by NaCl even when it is added some time after oxygen exposure indicates that in *T. tubifex* the effect of oxygen is at least partially reversible. The enhanced survival of *T. tubifex* in 0.05 *M* NaCl after heating or exposure to hydrogen peroxide suggests that increased salinity may be effective in protecting this organism against other environmental stresses.

I wish to express my deepest appreciation to Dr. Howard S. Ducoff for his advice and guidance throughout this study, to Dr. John D. Anderson for his continued interest and to the Department of Physiology and Biophysics for the use of their facilities.

## Summary

1. One per cent glucose when present during oxygen exposure provided significant protection of *T. tubifex* from doses which resulted in high worm mortality.

2. Glucose protection was not reversed by 2,4-dinitrophenol; 2,4-dinitrophenol was itself protective.

3. Sodium chloride at a concentration osmotically equivalent to one per cent glucose provided protection equal to or better than that of glucose.

4. Proteose-Peptone, $CaCl_2$, KCl, $CoCl_2$, $MnCl_2$ and $NaNO_3$ also provided various degrees of protection when they were present during oxygen exposure.

5. The solute concentration providing maximum protection against oxygen poisoning had an optimum at a concentration osmotically equivalent to one per cent glucose.

6. Sodium chloride increased the survival of oxygen-exposed *T. tubifex* even when it was added as long as 12 hours after oxygen treatment.

7. Sodium chloride provided at least partial protection against the stress of heating or hydrogen peroxide exposure.

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